Gene Related to Anergy in Lymphocytes (GRAIL) Expression in CD4+ T Cells Impairs Actin Cytoskeletal Organization during T Cell/Antigen-presenting Cell Interactions*§

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GRAIL (gene related to anergy in lymphocytes), is an E3 ubiquitin ligase with increased expression in anergic CD4+ T cells. The expression of GRAIL has been shown to be both necessary and sufficient for the induction of T cell (T) anergy. To date, several subsets of anergic T cells have demonstrated altered interactions with antigen-presenting cells (APC) and perturbed TCR-mediated signaling. The role of GRAIL in mediating these aspects of T cell anergy remains unclear. We used flow cytometry and confocal microscopy to examine T/APC interactions in GRAIL-expressing T cells. Increased GRAIL expression resulted in reduced T/APC conjugation efficiency as assessed by flow cytometry. Examination of single T/APC conjugates by confocal microscopy revealed altered polymerization of actin and LFA-1 to the T/APC interface. When GRAIL expression was knocked down, actin polarization to the T/APC interface was restored, demonstrating that GRAIL is necessary for alteration of actin cytoskeletal rearrangement under anergizing conditions. Interestingly, proximal TCR signaling including calcium flux and phosphorylation of Vav were not disrupted by expression of GRAIL in CD4+ T cells. In contrast, interrogation of distal signaling events demonstrated significantly decreased JNK phosphorylation in GRAIL-expressing T cells. In sum, GRAIL expression in CD4+ T cells mediates alterations in the actin cytoskeleton during T/APC interactions. Moreover, in this model, our data dissociates proximal T cell signaling events from functional unresponsiveness. These data demonstrate a novel role for GRAIL in modulating T/APC interactions and provide further insight into the cell biology of anergic T cells.

Anergy describes a functional state of T cells (T) that is characterized by failure to proliferate or produce interleukin-2 (IL-2) following presentation of cognate antigen in a known stimulatory fashion. The induction of T cell anergy is an active process dependent upon coordinated up-regulation and degradation of multiple proteins (1–4). Following engagement of the T cell receptor, a distinct biochemical signature has been described in anergic CD4+ T cells. In contrast to naïve T cells, anergic T cells demonstrate diminished influx of calcium, impaired PLC-γ activation, diminished ERK and JNK phosphorylation, and impaired translocation of the transcription factor AP-1 to the nucleus (5, 6). Recent work suggests that T/APC interactions are distinct in anergic CD4+ T cells (5, 7). However, the precise mechanisms that regulate this interaction remain poorly understood.

Multiple anergy-related E3 ubiquitin ligases, including Cbl-b, Itch, and GRAIL, are up-regulated during, and required for, the induction and maintenance of T cell anergy (5, 8–11). GRAIL mRNA and protein expression are uniquely up-regulated in anergized CD4+ T cells and Foxp3+ CD25+ regulatory T cells (8, 12, 13). GRAIL expression is necessary for the induction of T cell anergy, and ectopic expression of GRAIL in T cells is sufficient for the induction of anergy and suppressor function (11, 14). It is clear that expression of GRAIL in T cells significantly alters proliferative capacity; however, the impact of GRAIL expression on T/APC interactions and signaling events associated with engagement of the TCR has not been fully elucidated. A better understanding of the mechanisms governing T cell activation and T/APC interactions continues to be an area of intense investigation as novel targets for immune control are likely to be realized. In this study, we examine the role of GRAIL expression in modulating T cell signaling and T/APC interactions.

EXPERIMENTAL PROCEDURES

Animals—BALB/c mice were obtained from Harlan (Madison, WI). DO11.10 mice were bred under specific pathogen-free conditions at the University of Wisconsin animal facility (Madison, WI). The Animal Care and Use Committee at the University of Wisconsin approved all experimental protocols involving use of mice.

Antibodies—The following antibodies were used for Western blot analysis and immunofluorescence: JNK1 (1:100, clone C17), phospho-JNK (1:200, clone G7) and phospho-Vav (1:500, clone Tyr 177) were purchased from Santa Cruz Biotechnology cell receptor; IRES, internal ribosome entry site; ICAM, intercellular adhesion molecule; APC, antigen-presenting cell.
(Santa Cruz, CA); phospho-44/42-ERK antibody (1:1,000, Cell Signaling Technology, Danvers, MA), ERK antibody (1:1,000; Millipore, Billerica, MA), Vav1 antibody (1:500, Upstate, Lake Placid, NY), and TRITC-phalloidin (1:500, Invitrogen, Carlsbad, CA).

**T Cell Culture and Retroviral Transduction**—CD4+ T cell lines were derived as described previously (11). Cells were restimulated every 8–14 days with T cell-depleted irradiated splenocytes, peptide, and IL-2. Retroviral transduction was performed approximately as described previously (15). Briefly, 24 hours after stimulation, spinfection (750 × g, 1 h, 32°C) was used with 8 μg/ml protamine sulfate plus 10 units/ml recombinant IL-2 to transduce T cells with retrovirus containing green fluorescent protein (GFP) or GRAIL-IREs-GFP. Retrovirus was produced as described previously (8). After 8 days, GFP+ T cells were FACS-purified and restimulated for expansion. Cells were used on day 5–14 post restimulation for all experiments. LUCHp and GRAILhp small interfering RNA knockdown vectors were generously provided by Dr. Edward Pearce and are described elsewhere (13). Eight days after transduction, cells were stained with anti-human CD8, sort-purified, and expanded.

**Flow Conjugation**—Daudi B cells were used as APCs and labeled with PKH-26 (Sigma), washed, and incubated with 1 μg/ml staphylococcal enterotoxin E for 1 h in T cell media containing 0.05% fetal bovine serum (FBS). Jurkat T cells were nucleofected using AmxA with constructs containing GFP or GRAIL-IREs-GFP. Forty-eight hours after nucleofection, dead cells were removed by centrifugation on Ficoll, and cells were washed twice. GFP-only or GRAIL-IREs-GFP-expressing Jurkat T cells were incubated 1:1 with Daudi cells for 15 min in T cell media containing 5% FBS, fixed with an equal volume of 6% isonicotinic paraformaldehyde, and then vortexed for 30 s. Conjugates were quantified by flow cytometry and conjugation efficiency was measured as the number of double positive (red/green) events divided by the sum of the double positive events plus the unconjugated GFP+ cells.

**Calcium Flux**—GFP or GRAIL-IREs-GFP-expressing DO11.10 T cells were loaded with 5 μM Indo-1-AM (Invitrogen), washed twice, and coated with biotinylated anti-CD3 (0.1–1.5 μg/ml, clone 145-2C11; eBioscience, San Diego, CA). Baseline fluorescence was recorded for 30 s, streptavidin (10 μg/ml, Sigma) was added for cross-linking, and calcium flux was assayed by measuring the fluorescence emission ratio at 405/495 nm with FACS.vantageSE (BD Biosciences) equipped with a FACS-DeVio digital upgrade and was analyzed using FlowJo software (Tree-star, San Carlos, CA).

**Immunoblot**—Following centrifugation on a lymphoprep gradient (CedarLane Labs, Burlington, NC), DO11.10 T cells were washed twice and incubated with 1–1.5 μM ionomycin in T cell media for 16 h at 37°C. Cells were then washed twice in T cell media and rested for 2 h at 37°C, and 104 T cells were co-cultured with 2 × 105 irradiated BALB/c splenocytes pulsed with 0.4 μg/ml OVA-(323–339) peptide. Cells were pulsed with 1 μCi of [3H]-thymidine for the last 16 h of a 72-h co-culture.

**Real-time Quantitative PCR**—Four h after the addition of ionomycin to cultures, RNA from control or ionomycin-treated T cells was isolated using TRIzol reagent according to the manufacturer’s instructions (Invitrogen) and treated with RNase-free DNase I (Ambion). Real-time PCR was performed as described previously (14, 17).

**Microscopy**—A20 antigen-presenting cells were labeled with Cell Tracker Blue (Invitrogen) and loaded with OVA-(323–339) peptide in medium containing 0.1% FBS at 37°C for 1–2 h. A20 cells and either GFP- or GRAIL-IREs-GFP-expressing DO11.10 T cells were combined at a 1:1 ratio, centrifuged for 5 min at 500 rpm, and allowed to interact at 37°C for 15 min in T cell medium containing 5% FBS. Medium was removed, pellets were resuspended in pre-warmed PBS with calcium and magnesium, and cells were allowed to adhere to poly-l-lysine coated coverglass for 5 min. For studies using ionomycin-treated T cells, DO11.10-GFP expressing T cells were incubated with 1–1.5 μM ionomycin for 16 h, washed twice, and allowed to recover for 2 h at 37°C before interaction with A20 cells. Immunofluorescence was performed as described previously (18). Conjugates were visualized with a Nikon TE300 epifluorescence microscope using a Nikon 100× objective (numerical aperture = 1.4) where images were captured using a charge coupled device camera (Hamamatsu Photonics) and manipulated with MetaMorph software (Universal Imaging). Alternatively confocal images were acquired using a Nikon Eclipse TE2000 with a 60× objective run on a Dell OptiPlex computer with dual displays running LaserSharp 6.0. Single T/APC conjugates were extracted, resolved on SDS-PAGE, and transferred to polyvinylidene difluoride. Antibody binding was detected using horseradish peroxidase-conjugated anti-rabbit IgG (Sigma) and the ECL+ Western blot detection system (Amersham Biosciences). Bands were detected using a Storm PhosphorImager (Amersham Biosciences) and quantified using ImageQuant (Molecular Dynamics).

**Adhesion Assay**—Adhesion assay performed essentially as described (16). Briefly, 96-well high protein binding plates (Greiner Bio-One) were coated for 3 h with 3 μg/ml rmlCAM-1 (R&D Systems) and blocked for 1 h with 1% bovine serum albumin. Cells were labeled with 0.5 μg/ml Calcein-AM (Invitrogen) for 15 min at 37°C, washed, and resuspended in RPMI supplemented with 5% FBS. Cells were left unstimulated or stimulated with 0.5 μg biotinylated anti-CD3 (eBioscience) and 1 μg streptavidin per million cells, 20 ng/ml PMA, and 1 mM MnCl2, and allowed to adhere to plate for 25 min at 37°C. Following incubation, fluorescence was measured using a Victor plate reader (PerkinElmer Life Sciences) and % adhesion calculated as the ratio of fluorescence after washing to initial fluorescence. Values are mean ± S.E. of three experiments.

**Ionomycin Treatment and Proliferation**—Following centrifugation on a Lymphoprep gradient (CedarLane Labs, Burlington, NC), DO11.10 T cells were washed twice and incubated with 1–1.5 μM ionomycin in T cell media for 16 h at 37°C. Cells were then washed twice in T cell media and rested for 2 h at 37°C, and 104 T cells were co-cultured with 2 × 105 irradiated BALB/c splenocytes pulsed with 0.4 μg/ml OVA-(323–339) peptide. Cells were pulsed with 1 μCi of [3H]-thymidine for the last 16 h of a 72-h co-culture.

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imaged, and actin polarization was scored from 1–5 as shown in supplemental Fig. S1.

**Statistical Analysis**—Statistical analyses were calculated using the two-tailed Student’s *t* test. Values ≥ 0.05 were considered statistically significant.

**RESULTS**

Expression of GRAIL in CD4+ T Cells Leads to Disrupted Actin Cytoskeletal Organization during T/APC Interactions—Unstable immunologic synapse formation has been described previously in anergized T cells. However, actin polarization has not been directly investigated in anergic CD4+ T cells (5). Therefore, we sought to characterize actin distribution in CD4+ T cells anergized via exposure to ionomycin. Ionomycin-mediated T cell anergy is a well characterized model for the induction of increased expression of GRAIL mRNA and protein in CD4+ T cells (5, 8). Similar to previous reports, ionomycin treatment resulted in up-regulation of GRAIL mRNA expression and significant abrogation of DO11.10 T cell proliferation in response to OVA-(323–339)-pulsed APCs (data not shown) (5, 8). Control and ionomycin-treated DO11.10 T cells were allowed to interact with OVA-(323–339) pulsed A20 B cells for 15–20 min and examined using confocal microscopy as described under “Experimental Procedures.” Single T/APC conjugates were then scored as described in supplemental Fig. S1. Data are pooled from three independent experiments.

**FIGURE 1.** Ionomycin induced T cell anergy leads to up-regulation of endogenous GRAIL and altered actin organization during T cell/APC interactions. *A,* sixteen h after ionomycin treatment, control or ionomycin-treated T cells were allowed to interact with OVA-(323–339)-pulsed A20 cells, and immunofluorescence was performed as described under “Experimental Procedures.” Confocal images were obtained, and single T/APC conjugates were examined for actin polarization to the T/APC interface. Images from two separate experiments are shown. *B,* actin polarization to the T/APC interface was then scored as described in supplemental Fig. S1. Data are pooled from three independent experiments. The median polarization score is shown in red. *C,* DO11.10 T cells expressing LUCrp or GRAILhp were treated with ionomycin. Quantitative real-time PCR was performed to examine GRAIL mRNA expression 3 h after treatment. *D,* confocal images were obtained and single T/APC conjugates were examined for actin polarization to the T/APC interface as in *A.* Data are pooled from two independent experiments. CMAC, 7-amino-4-chloromethylcoumarin.

**FIGURE 2.** GRAIL-expressing T cells demonstrate perturbed cytoskeletal organization during T cell/APC interactions. *A,* DO11.10 T cells expressing GFP or GRAIL-IRES-GFP were allowed to interact with OVA-(323–339)-pulsed A20 cells for 15 min, and immunofluorescence was performed as described under “Experimental Procedures.” Confocal images were obtained and single T/APC conjugates were examined for actin polarization to the T/APC interface. *B,* actin polarization to the T/APC interface was then scored as described in supplemental Fig. S1. The median polarization score is shown in red. Data are representative of a minimum of five independent experiments. CMAC, 7-amino-4-chloromethylcoumarin.
Actin polarization during T/APC conjugation was examined using untreated or ionomycin-treated T cells expressing either LUC.hp or GRAIL.hp. Ionomycin treatment of LUC.hp-expressing T cells resulted in reduction in the median actin polarization score from 2 to 0 (mean ± S.E. 2.04 ± 0.21 compared with 0.92 ± 0.17). In contrast, the median polarization score was unchanged in GRAIL.hp-expressing T cells following treatment with ionomycin (mean ± S.E. 2.07 ± 0.23 compared with 1.91 ± 0.22) (Fig. 1D). Together, these findings demonstrate that GRAIL is necessary for the observed impaired actin polarization in anergic T/APC conjugates.

Next, we examined T/APC interactions in T cells with forced expression of GRAIL. GFP control or GRAIL-expressing DO11.10 T cells were allowed to interact with OVA-(323–339)-pulsed A20 cells for 15 min. Actin polarization to the T/APC interface was examined using epifluorescence microscopy and quantified as above. GFP expressing T/APC conjugates were scored at ≥4 in 48% of scored conjugates, indicating nearly complete actin cap formation. In contrast to control T cells, GRAIL-expressing T cells had significantly impaired polarization of actin to the T/APC interface (Fig. 2A). GFP-expressing control T cells had a median polarization score of 3.5 (mean ± S.E., 3.12 ± 0.31) compared with 1 (1 ± 0.25) for GRAIL-expressing T cells with the median score identified in red (Fig. 2B). While some partial polarization was observed, formation of a complete actin cap was not identified in any GRAIL-expressing T cells.

Disruption of the actin cytoskeleton has been reported to reduce T cell conjugation efficiency (19). To determine whether GRAIL expression affects conjugation efficiency, we used flow cytometry to quantify T/APC conjugate formation in GFP- or GRAIL-expressing Jurkat T cells. Jurkat T cells were allowed to interact with staphylococcal enterotoxin E-pulsed Daudi B cells for 15 min. Although nearly 30% of GFP-expressing T cells formed conjugates with APCs, conjugation efficiency was reduced ~2-fold in GRAIL-expressing T cells with only 14% of GRAIL-expressing T cells forming stable conjugates (Fig. 3). Interestingly, GRAIL expression was associated with reduced conjugate formation in the absence of cognate antigen, suggesting there may be a TCR-independent effect in this context (6.3% versus 3% conjugation, GFP versus GRAIL, respectively; Fig. 3). These findings are consistent with previous reports showing that anergic T cells have disrupted APC interactions and together suggest that GRAIL expression is suffi-
cient to alter T/APC interactions and disrupt cytoskeletal organization in CD4+ T cells.

**LFA-1 Polarization, But Not Adhesion to ICAM, Is Impaired in GRAIL-expressing T Cells**—The functional status of LFA-1 contributes to T cell adhesion and activation (20, 21). In addition, anergized T cells are reported to have altered LFA-1 polarization (5). We sought to determine whether GRAIL expression impacted either LFA-1 polarization during T/APC interactions or T cell adhesion to ICAM. Whereas robust polarization of LFA-1 was observed in control T cells during interaction with OVA-(323–339)-pulsed A20 cells, GRAIL expression in T cells resulted in a patchy distribution of LFA-1 during T/APC interactions and impaired LFA-1 polarization to the T/APC interface (Fig. 4A). Next, we examined LFA-1 expression by flow cytometry and function by quantifying TCR-mediated adhesion to plate-bound ICAM. Neither surface expression of LFA-1 (data not shown) nor adhesion to ICAM was affected by expression of GRAIL, indicating that LFA-1 expression and function are not diminished in GRAIL-expressing T cells (Fig. 4B). These findings demonstrate that in GRAIL-expressing T cells, the lack of actin polarization is linked with the absence of LFA-1 polarization; however, LFA-1/ICAM interactions are not disrupted.

**GRAIL-expressing T Cells Have Intact Proximal TCR Signaling**—Actin cytoskeletal remodeling is integral for the coordination of sustained TCR signaling events, including mobilization of calcium (19, 22, 23). We examined calcium mobilization in GFP- or GRAIL-expressing DO11.10 T cells following engagement of the TCR. Calcium influx was unaffected and was similar in the GFP- and GRAIL-expressing T cells after cross-linking of the TCR (Fig. 5A). In addition, we assayed calcium mobilization under more physiologic conditions, during T/APC interactions, and observed similar results to cross-linking of the TCR data (data not shown).

Coordination of cytoskeletal dynamics during T cell activation is regulated by an extensive family of regulatory proteins that promote stabilization of the T/APC interaction (24). In T cells, this coordination is accomplished in part by the guanine exchange factor Vav1. Vav1 is recruited to the immunologic synapse and is required for optimal calcium flux, actin polymerization and cap formation, IL-2 production, and proliferation (25–27). To interrogate phosphorylation of Vav, cells were serum-starved for 2 h and stimulated via cross-linking of the TCR. Somewhat surprisingly, there was no difference in the level of phosphorylated Vav in the GFP- and GRAIL-expressing T cells under these conditions (Fig. 5B).

**JNK Phosphorylation Is Diminished in GRAIL-expressing T Cells**—Proliferation and IL-2 production in T cells are dependent upon phosphorylation of the mitogen-activated protein kinases ERK and JNK (6, 28). As both T cell proliferation and IL-2 production are inhibited by GRAIL expression, we examined ERK and JNK phosphorylation by immunoblot analysis following activation of GFP- or GRAIL-expressing DO11.10 T cells. GRAIL- and GFP-expressing T cells demonstrated robust and comparable phosphorylation of ERK within 5 min of activation (9-fold versus 12-fold increase compared with resting, respectively, Fig. 6A).

Efficient induction of JNK phosphorylation was observed in GFP-expressing T cells within 15 min of cross-linking of the TCR.
GRAIL Alters Actin Organization and JNK Phosphorylation

In contrast, GRAIL-expressing T cells demonstrated nearly complete abrogation of JNK phosphorylation in response to activation (6.9-fold versus 1.2-fold, Fig. 6B). These findings suggest that GRAIL expression induces impaired signaling through JNK but not ERK mitogen-activated protein kinases. Moreover, the overall tyrosine phosphorylation pattern (data not shown) was generally normal in GRAIL-expressing T cells, indicating that GRAIL-associated T cell anergy is an active and specific process.

**DISCUSSION**

In this study, we have demonstrated a novel role for GRAIL in regulating cytoskeletal organization in T cells. Ionomycin treatment of T cells results in a GRAIL-dependent disruption of actin polarization to the T/APC interface. In addition, ectopic expression of GRAIL results in impaired actin polarization during T/APC interactions. GRAIL-expressing T cells revealed a specific disruption in JNK phosphorylation in the presence of intact proximal signaling and normal ERK phosphorylation. This is in contrast to other anergy-related E3 ligases, such as Cbl-b and Itch, where more proximal TCR signaling molecules have been reported to be affected (5, 9, 10). These findings provide new insights into the cell biology and molecular mechanisms that contribute to CD4+ T cell anergy. The relationship between diminished JNK phosphorylation and disrupted actin cap formation is still being investigated.

Anergic T cells have been reported to form unstable immunologic synapses. However, examination of actin organization has not been described previously (5). In this study, we demonstrate disrupted actin organization in anergic T cells that is dependent on endogenous GRAIL expression and can be recapitulated with ectopic expression of GRAIL. Although the precise contribution of actin cytoskeletal remodeling to T cell signaling and biologic function is not completely understood, reorientation of the actin cytoskeleton to the T/APC interface, known as actin cap formation, is considered important for T cell activation (29). Moreover, disruption of the actin cytoskeleton or several critical actin regulatory proteins has been shown to perturb T cell activation (22, 26). It is interesting that the actin cytoskeletal phenotype observed in our studies is similar to phenotypes reported for T cells lacking any of several actin regulatory proteins including Vav1 (22, 26). However, expression of GRAIL was not associated with a decrease in expression or phosphorylation of Vav1. Although this early actin regulatory protein appeared normal in our model, our data does not preclude the disruption of any number of distal events necessary for the coordinated cytoskeletal remodeling. Our data further support a role for the actin cytoskeleton in contributing to T cell function and suggest that GRAIL expression in T cells may regulate cytoskeletal organization in a Vav-independent manner.

Coincident with perturbed actin polarization, GRAIL-expressing T/APC interactions exhibited altered polarization of LFA-1 to the contact interface. Impaired LFA-1 polarization to the T/APC interface has been associated with reduced conjugation efficiency, impaired LFA-1 signaling, and diminished adhesive function and is linked to actin cytoskeleton polarization (16, 30–32). Our data demonstrate that GRAIL expression impaired actin and LFA-1 polarization and conjugation efficiency; however, LFA-1 adhesive function was not impacted by expression of GRAIL. Interestingly, CD25+Foxp3+CD4+ T regulatory cells, a T cell with increased expression of GRAIL (14), impaired actin cap formation (33), and anergic features (34) are capable of binding to ICAM on APCs and appear to be dependent upon this receptor/ligand interaction for suppressor function (35, 36). Our data suggest that diminished T/APC conjugation in GRAIL-expressing T cells is likely not due to grossly altered LFA-1 function and suggests a unique function for GRAIL in perturbing actin cytoskeletal and LFA-1 remodeling or kinetics while permitting LFA-1 function.

In addition to actin cytoskeletal rearrangement and characteristic interactions with APCs, T cell activation is dependent upon defined intracellular signaling cascades that are linked to biologic function. An early biochemical event that occurs after engagement of the TCR on a T cell is increased intracellular levels of calcium that facilitate downstream biochemical signaling cascades. Although the reciprocal regulation of calcium and

**FIGURE 6.** GRAIL-expressing T cells have diminished JNK phosphorylation. DO11.10 T cells expressing GFP or GRAIL-IRES-GFP were incubated in serum-free media for 2 h at 37°C and left untreated or coated with biotinylated anti-CD3 followed by cross-linking with streptavidin for 5 min. Cell lysates were subjected to electrophoresis. Blots were then probed with antibodies to phosphorylated ERK (A) or phosphorylated JNK (B), stripped, and reprobed with antibodies to total ERK or JNK (left). Band intensities were quantified and are displayed as normalized band volumes (right). Data are representative of a minimum of three experiments.
GRAIL Alters Actin Organization and JNK Phosphorylation

the actin cytoskeleton has not been fully delineated, current data suggest calcium influx promotes the polymerization of actin necessary for cytoskeletal polarization of F-actin to the T/APC interface (37). Anergic T cells are generally characterized by diminished calcium flux following activation (5, 28, 38). The molecular regulation for this is at least partially dependent upon up-regulation of the E3 ubiquitin ligase Cbl-b (9). Somewhat surprisingly, calcium flux was normal in GRAIL-expressing T cells during engagement of the TCR or during interaction with APCs. Thus, our data does not support a role for GRAIL expression in mediating diminished calcium flux in anergic T cells and suggests that actin polarization and calcium flux can be dissociated in our model. Previously published work has demonstrated impaired IL-2 production and proliferation in response to TCR engagement in GRAIL-expressing T cells (8, 11). Multiple biochemical mediators, including phosphorylation of mitogen-activated protein kinase family members ERK and JNK, are reported to be disrupted in anergic T cells (6, 28, 38, 39). In this study, demonstration of diminished JNK phosphorylation in GRAIL-expressing T cells is consistent with published data in anergicized T cells following cross-linking of the TCR. However, the specific disruption of JNK phosphorylation in the presence of intact ERK phosphorylation has not been described previously in anergic T cells (6). The role of JNK phosphorylation in T cell activation remains controversial with some reports suggesting JNK may be dispensable for T cell proliferation and cytokine production (40, 41). Nonetheless, in several model systems, blockade of JNK activation has been shown to abrogate IL-2 production and proliferation in T cells (42, 43). Biochemical signaling in response to stimulation with PMA/ionomycin was previously interrogated in GRAIL-expressing Jurkat T cells, and JNK phosphorylation was comparable to the control Jurkat T cells (44). This disparity can be reconciled by the fact that following activation with PMA/ionomycin JNK phosphorylation is intact in anergized T cells (6). Furthermore, in our hands, the GRAIL expressing T cells can be expanded in vitro using co-stimulation and exogenous IL-2, and these conditions are linked to down-regulation of GRAIL expression (data not shown). Therefore, our data support a critical role for TCR-mediated signals in maintaining the biochemical signature of anergic T cells and suggests that under certain contexts, for example PMA/ionomycin or perhaps strong co-stimulation during physiologic contexts, this signature can be overridden.

This study demonstrates that ectopic expression of GRAIL and up-regulation of endogenous GRAIL via ionomycin treatment of T cells results in actin cytoskeletal rearrangement and distinct biochemical signaling pathway alterations. These data suggest that one functional role for GRAIL in T cells may be to regulate the actin cytoskeletal interaction upon an encounter with an APC. While the biologic implications of these observations are not yet completely clear, there is accumulating evidence supporting a complexity of immune regulation occurring via T:APC interactions for naïve, effector, anergic, and regulatory T cells (45–47). We propose that expression of GRAIL in T cells may modulate T:APC interactions, thereby impacting functions ranging from homeostasis and proliferation to cytokine secretion and survival. T cell responses is the ultimate goal of immune-based therapies for a multitude of human diseases, and regulation of GRAIL expression may provide a novel target for control of T cell fate and biologic function.

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